Food flours with specific technological characteristics and low allergenicity

Field of the art

The present invention refers to the technology for the realization of a new cereal from which seed a food flour can be obtained, and from which dough bakery products can be obtained.

International Classification: C12 n, A01 b.

State of the art

The bakery products currently known are obtained almost exclusively from wheat flours. Such flours, due to the leavening property of their dough made of water and yeast, maintain an alveolar structure after baking in the oven.

Such a property gives wheat flours, especially when kneaded with water and yeast, the quality of forming a dough which is elastic enough to hold inside it the gas produced through fermentation and to develop a soft and elastic structure after baking.

High and low molecular weight glutenins, the main storage proteins of the wheat endosperm, are responsible for these particular technological properties. Glutenin's particular sequence enables it to interact in order to form a complex three-dimensional structure that can stretch and trap the carbon dioxide that develops in the leavening phase, thus giving the end product a high specific volume.

Many people are allergic to the gluten contained in wheat flours, in particular to the gliadine and glutenin components with low molecular weight, and thus require particular dietary attentions (Sollid, 2000).

The problem to be solved is to produce non-allergenic flours that nonetheless maintain the property causing them to rise, that is, which are able to form a dough that can be used to make bakery products and that has the same technological properties of dough obtained with wheat flours (Schuppan and Hahn, 2002).

The present invention suggests an optimal solution to this problem and allows non-allergenic, rising flours to be obtained.

Description

The invention will now be disclosed with reference, solely by way of example, to the technological process of endowing rice flours with the potential to generate a dough that can rise, is elastic and can be used to create bakery products with high specific volumes.

Rice is known to be a cereal with a very peculiar nutritional profile and is considered the most suitable food for children and the elderly.

Rice is in fact a hypoallergenic, highly digestible food, with a protein profile which shows little diversity but is of very high quality.

Rice's high digestibility is due to the small dimension of its starch granules, which are twenty times smaller than wheat's and seventy times smaller than the potato's.

Rice is the second cereal after wheat in terms of worldwide production: rice fields cover a hundred and fifty million hectares and produce five hundred million tons of rice each year.

Italy is the main European producer with around two hundred thousand cultivated hectares. Among major cereals, rice has the smallest genome, sixty times smaller than wheat's and twelve times smaller than corn's.

The rice genome, consisting of 12 chromosomes, is completely sequenced. The availability of the sequence of all the genes of this species makes it possible to study its storage protein components and allows its genetic complement to be modified utilizing regions of regulation-specific to the seed storage components.

The invention also concerns the construction of new expression plasmids that allow the production and accumulation - in the seed of cereals such as rice, corn and soybean - of storage proteins of wheat and enzymes of animal origin. The new expression plasmids allow the tissue-specific accumulation of proteins.

In the present invention the design and realization of the expression system in plants is documented, with the demonstration of its validity in a plant such as rice. In order to obtain the seed-specific expression of proteins, the promoters and signal sequences belonging both to the wheat genes and to the rice storage proteins genes were used.

These regulation and structural sequences were isolated and cloned from the wheat varieties Cheienne, Centauro, Golia, Pandas and Veronese. The gene for the animal enzyme transglutaminase was cloned starting from the cDNA of the liver tissue of a guinea pig. All the cloned gene components were controlled at the sequence level. The cloned sequences were used as such or after mutagenesis to

eliminate possible epitopes known as activators of the immune response in patients with gluten allergy.

The final constructs used for rice transformation, also usable in other cereals and in legumes, were realized in vectors of the pUC 19 type and with these, through co-transformation of the constructs with physical methods, immature embryos of Ariete and Rosa Marchetti rice cultivars were transformed. For each transformation experiment, performed using up to ten constructs in various combinations, 100 transgenic (T_0) plant resistant to hygromycin were selected and these were controlled at a molecular level with PCR techniques. The further combination of the genes of interest in a single transgenic line was realized through crossing, followed by diploidization of aploid lines, regenerated by an anthers culture, to reach the homozygosis state faster.

The specificity of accumulation of the various proteins in the seed was controlled with dot blot and Western techniques using polyclonal antibodies developed against the wheat proteins produced in *E. coli*.

Therefore the invention makes available: (1) new rice varieties characterized by an ability to accumulate different wheat storage proteins and an animal enzyme, also of human origin, able to foster the formation of interchain links between the proteins; (2) new plasmid vectors made for the production of wheat storage proteins in other cereals; (3) a rice flour with technological characteristics similar to the ones of wheat flour.

Another aspect of the invention includes the following components functionally linked by 5' and 3' to form a plasmid expression vector: (a) a promoter; (b) a nucleotide sequence corresponding to the aminoacid sequence of the wheat glutenin having a certain c-terminal sequence, or corresponding to guinea pig transglutaminase; c) a signal of polyadenilation.

The DNA sequences from (a) to (c) are cloned in different vectors to form plasmids. The resulting expression plasmids can be used to transform plant cells with direct physical methods. The transformed plant cells are selected and induced to form entire fertile plants that produce seeds able to express the genes of storage or enzymatic proteins.

Another key element of the invention includes nucleotide sequences of wheat glutenins that are modified with techniques of direct mutagenesis in order to eliminate aminoacid sequences known as allergenic in food allergies to gluten.

Another aspect of the invention regards the use of flours taken from seeds of plants transformed with the above mentioned plasmids, for the production of baked products, after kneading and fermentation.

Brief description of tables and figures.

The characteristics, elements and goals of the invention, briefly described earlier, will become much clearer and more understandable when illustrated with reference to tables and figures that follow. It should be noticed, however, that the examples in the figures show preferential elements of the invention and should not be considered as limiting its scope.

Table 1 (enlarged 1A-1B) shows the aminoacid sequences of the wheat proteins chosen for the expression in rice and the preserved C-terminal motive LKVAKAQQLAAQLPAMCR (position 945-962).

Table 2 shows the nucleotide sequence of the gene for the guinea pig transglutaminase enzyme.

Table 3 shows one of the nucleotide sequences of rice's regulation region used for the seed-specific expression of wheat and guinea pig genes.

Table 4 shows the oligo-nucleotide sequence used for the cloning of some wheat storage proteins genes and the guinea pig transglutaminase enzyme.

Table 5 shows the result of an ELISA test performed on wheat flour, rice flour and the new flour of the line PLT3000R13-7.

Figure 1 shows the plasmid pIGP 2001 obtained by cloning the wheat gene 1Bx7.

Figure 2 shows the plasmid pIGP 2002 obtained by cloning the wheat gene 1By9.

Figure 3 shows the plasmid pIGP 2003 obtained by cloning the wheat gene 1Dx5.

Figure 4 shows the plasmid pIGP 2004 obtained by cloning the wheat gene 1Dy10.

Figure 5 shows the plasmid pIGP 2005 obtained by cloning the wheat gene 1Ax2.

Figure 6 shows the plasmid pIGP 2006 obtained by cloning the wheat gene 1Bx17.

Figure 7 shows the plasmid pIGP 2008 obtained by cloning the wheat gene GluHMW2.

Figure 8 shows the plasmid pIGP 2009 obtained by cloning the wheat gene Glu1A.

Figure 9 shows the plasmid pIGP 2010 obtained by cloning the wheat gene 1Ax1.

Figure 10 shows the plasmid pIGP 2012 obtained by cloning the wheat gene 1Dy12.

Figure 11 shows the plasmid pIGP 2050 obtained by cloning the variant MUT1 of the wheat gene 1Dy10.

Figure 12 shows the plasmid pIGP 2051 obtained by cloning the variant MUT1 of the wheat gene 1By9.

Figure 13 shows the plasmid pIGP 2052 obtained by cloning the variant MUT3 of the wheat gene 1By9.

Figure 14 shows the plasmid pIGP 2100 obtained by cloning the gene that codes for guinea pig's transglutaminase (TG).

Figure 15 shows, by way of example, an agarose gel with the DNA resulting by amplification through PCR, performed using the specific primers for the single wheat's genes, on DNA extracted by T_0 rice plants transformed with the plasmids of figures 1-14. The agarose gel is colored with Ethidium bromide and photographed under UV light to highlight the amplification products obtained using DNA extracted from leaves of rice lines transformed with the pIGP2002 vector and two primers that amplify an internal fragment, about 300 pb, of the gene . M= markers of molecular weight (100 bp ladder Promega); C+ = positive control (plasmid DNA); 1-16 = single rice plants regenerated on selection medium; 17 = negative control (DNA extracted from a plant of the Rosa Marchetti variety). The positive plants are the ones that have the fragment indicated by an arrow.

Figure 16 shows, by way of example, the results of the Southern analysis performed on some T_1 plants of figure 15, transformed with the plasmids of figures 1-14. The Southern analysis is performed using DNA extracted from transgenic lines of rice positive to PCR. As a probe a fragment of the By9 gene was used, the genomic DNA of rice was cut with two enzymes in order to have an indication of the number of copies of the genes present in each line. 1-9 = transgenic lines of rice transformed with the pPGI2002 plasmid; C- = negative control (DNA of the Rosa Marchetti variety); C+ = positive control.

Figure 17 shows by way of example a SDS-PAGE gel of total proteins extract from the seeds of the indicated transgenic plants T_2 and their Western analysis, after transfer on membrane, using polyclonal antibodies specific for the wheat storage protein 1By9. The Western analysis is performed on total proteins, extracted from single seeds of transgenic rice, after separation through SDS-PAGE electrophoresis, transfer on membrane and detection in chemiluminescence using as primary antibody a polyclonal produced in rabbit and specific of the protein By9. W = total proteins extracted from the wheat seed; 1-10 = total proteins extracted from the seed of transgenic lines of rice in segregation; C+ = positive control (protein By9 produced in *E.coli*).

Figure 18 shows, by way of example, a SDS-PAGE gel of total proteins extract from the seeds of the indicated transgenic plants and Western analysis of the same, after transfer on membrane, using polyclonal antibodies specific for the guinea pig's transglutaminase (TG). The Western analysis is performed on total

proteins, extracted from single seeds of transgenic rice, after separation through SDS-PAGE electrophoresis, transfer on membrane and detection in chemiluminescence using as primary antibody a polyclonal produced in rabbit and specific of the protein transglutaminase (TG). 1-7 = total proteins extracted from the seed of some transgenic lines of rice; C+ = positive control (protein TG produced in *E.coli*); C- = negative control (proteins extracted from the Rosa Marchetti variety).

Figure 19 shows, by way of example, a SDS-PAGE gel of total protein extract from the rice transgenic lines transformed with the gene for the protein 1Dy10 and their Western analysis after transfer on membrane, using a specific polyclonal antibody. The Western analysis is performed on total proteins, extracted from single seeds of transgenic rice, after separation through SDS-PAGE electrophoresis, transfer on membrane and detection in chemiluminescence using as primary antibody a polyclonal produced in rabbit and specific of the Dy10protein. W = total proteins extracted from wheat seed; 1-11 = total proteins extracted from seeds of different transgenic lines of rice; C+ = positive control (Dy10 protein produced in *E.coli*); C- = negative control (proteins extracted from the seed of Rosa Marchetti variety).

Figure 20 shows, by way of example, a one-dimensional electrophoresis of the storage proteins of some wheat cultivars, in which the bands corresponding to the cloned genes are highlighted. Staining with comassie blu of a SDS-PAGE gel highlights the high molecular weight glutenins in the indicated varieties and used, with other varieties, in the cloning work of the single corresponding genes.

Figure 21 shows, by way of example, a one-dimensional electrophoresis of the wheat storage proteins where the high molecular weight class and the low molecular weight class of glutenins are visible. Staining with comassie blu of a SDS-PAGE gel highlights the high molecular weight glutenins (higher part of the gel) and the low molecular weight glutenin (lower part of the gel) present in 9 cultivars of bread wheat.

Figure 22 shows the result of a Western analysis performed on the proteins of figure 21, after transfer on membrane, using the serum of a patient with gluten allergy, to highlight the almost exclusive recognition of the low molecular weight glutenins. The Western analysis is performed on total proteins of figure 21, after transfer on membrane and detection in chemiluminescence using as primary antibody the IgA + IgG of the serum of a celiac patient.

Figure 23 shows, by way of example, the result of a bread-making test in which the dough was prepared using flour produced by a transgenic line of rice (on the right) that expresses the wheat proteins 1Ax1, 1Dx2, 1Dx5, 1Bx6, 1Bx7, 1Bx17, MUT11Dx10, MUT11By9 and the enzyme TG, compared with a normal rice flour (on the left).

Figure 24 shows, by way of example, the result of the test described in the figure 23 to show the alveolar form and the rising obtained with the new flour compared to a normal rice flour.

Figure 25 shows, by way of example, the alveogram obtained with the new flour produced with the seed of the line reported in figure 23. It shows also the results of

the alveogram performed on the dough obtained from the flour of table 5. The results are P/L = 0.78 mmH2O/mm e W = 28 E-4J.

Figure 26 shows, by way of example, the result of a PCR analysis aimed at demonstrating the presence of the gene for the transglutaminase enzyme in the transformed lines. The agarose gel is stained with Ethidium bromide and photographed under UV light to highlight the amplification products obtained using DNA extracted from leaves of rice lines transformed with the pIGP2100 vector and two primers that amplify the gene of about 2070 pb. 1kb = molecular weight markers; P+ = positive control (plasmid DNA); B = negative control (DNA extracted from a plant of the Rosa Marchetti variety). The plants represent the progeny of some transformed lines.

Detailed description of the invention.

For the cloning of the sequences corresponding to the glutenin genes of high molecular weight, of wheat, with or without the regulation region, the polymerase chain reaction (PCR) technique was used, starting from the information sequences present in the databank. Genomic DNA extract from the leaves of Triticum Aestivum cultivar Cheienne, Chiarano, Centauro, Golia, Pandas and Veronese was used. Some of the oligonucleotides used for the specific amplification are reported in table 4.

For the cloning of the sequence corresponding to the guinea pig's gene that codes for the transglutaminase enzyme, the RT-PCR technique was used. In this case

total RNA extract from guinea pig's liver was used and the amplification specific oligonucleotides are reported in table 4.

Once cloned, the genes that code for the wheat proteins were used as such or after site-direct mutagenesis to replace specific aminoacids.

Specifically, the modified nucleotide sequences code for the aminoacid sequences of the type - as a non-restrictive example - PFPQPQLPY, PQPQLPYPQ, PYPQPQLPY, LQLQPFPQPQLPY, QQGYYPTSPQQSG, QQGYYPTS, PFSQQQQQ, QSEQSQQPFQPQ and QXPQQPQQF paying special attention to the replacement of the glutamine and of the other aminoacids in underlined positions (Willemun et al., 2002; Shan et al., 2002).

For the rice promoter PROL we started from sequence information gained from experiments of differential display that highlighted the specificity of expression in the seed of the original clone. After the comparison of the obtained sequence with the databank the clone resulted matching 100% with the sequence with Acc. Number AF156714, and from this we started cloning, using the PCR technique from the genomic DNA of Ariete variety.

The wheat amplification products correspond to the expected dimensions for the specific genes according to the EMBL sequence data.

In the case of the rice promoter, the template DNA was extracted from the leaves of *Oryza Sativa* cultivar Ariete and the product of the amplification corresponds to the expected dimensions according according to the EMBL sequence data.

Starting from the amplified fragments, through ligation in the vector pGEM-T, the vectors were built from which the single fragments were recuperated, using the indicated enzymes, to insert them in the vector pPLT 100 (derived from pUC19) to obtain the final constructs shown in figures 1-14.

The final plasmids were verified through restriction analysis using different enzymes and one clone for each type was chosen and sequenced. The sequenced clones turned out to be identical to the sequences present in the databank, with the exception of the sequence of the promoter PROL, which shows some nucleotide differences compared to the sequence in the databank.

The plasmids pIGP2001, pIGP2002, pIGP2003, pIGP2004, pIGP2005, pIGP2006, pIGP2008, pIGP2009, pIGP2010, pIGP2012, pIGP2050, pIGP2051, pIGP2052, pIGP2100 and pIGP2500 (which carries the hygromycin resistance gene used for the selection of the transformed) were purified from cellular cultures of *E. coli* and the DNA utilized for the transformation of rice embryos with biolistic technique.

The T_0 plants were verified through PCR analysis (figure 15), the T_1 plant through Southern analysis (figure 16) and the T_2 plant, and following generations, through Western analysis (figures 17, 18 and 19).

The PCR positive plants show the accumulation of the corresponding protein, recognized by the specific antibody, only in the seed.

The presence of the recombinant protein only in the seed and not in the leaves was verified in all the examined transgenic plants.

Example 1: cloning of the genes that code for wheat proteins.

The genes of interest were cloned starting from genomic DNA of wheat extracted from single varieties known as having a good expression of the protein of interest. The bread wheat Cheienne, Chiarano, Centauro, Golia, Pandas and Veronese were mainly used. The genomic DNA was used as the template in PCR reactions that had to be optimized for each single gene (Mullis and Faloona, 1987). As an example, the conditions applied for the amplification of the gene Ax1 are reported here: initial denaturation at 98° C for three minutes, followed by 38 cycles of denaturation at 95° for one minute, annealing at 62° for one minute, extension at 72° for four minutes, followed by a final synthesis at 72° for ten minutes. The primers used were drawn for each single gene (table 4) considering both the structural part by itself, from the ATG to the stop codon, and the structural part plus the regulation region in 5' and in 3'.

The amplified fragments were cloned in the vector pGEM-T (Promega), sequenced and subcloned in vectors for expression in *E.coli* (pET 28a, Novagen) to produce the protein to be used in the immunization of rabbits, and in vectors for specific expression in rice (pPLT 100). In cases in which the genes were modified, they underwent several cycles of mutagenesis performed in the vector pGEM, followed by a further sequencing to verify the variations introduced in the codons.

Example 2: genetic transformation of rice embryos.

The plants of the rice varieties Ariete and Rosa Marchetti, chosen for the genetic transformation, were seeded in a greenhouse in March.

At flowering, the single spikleets were labeled indicating the exact date of flowering and after 11 days the immature embryos were excised from the seed, in sterile conditions, for genetic transformation with physical methods with the instrument PDS-1000/He (BioRad). The genetic modification was performed using a co-transformation technique where the selection marker (resistance to hygromycin) was present on a plasmid (pIGP 2500) separate from those containing the genes of interest (pIGP 2001 to 2100).

In the transformation experiments the total concentration of DNA was 1 μg/μl, using 0.6 μg of DNA for each shooting of target tissue. The ratio between the DNA with the selection marker and the DNA with the gene, or genes, of interest was 1:5 (calculated on the number of molecules). When the transformation included several genes of interest the ratio remained constant between the selection plasmid and the plasmids with the gene of interest (1:5), while the genes of interest remained in a 1:1 ratio with one another (e.g. for 6 genes the final molar ratio was 1:5:5:5:5:5:5). The transformation was performed transferring the marker plasmid in combination with a single plasmid or with several plasmids (up to 10) with the genes of interest (Chen et al., 1998).

In the case of transformation with one or few genes of interest, or when the molecular analysis highlighted the presence of only some of the introduced genes, the transgenic lines obtained were crossed to combine different genes in a single line. The segregating plants, which displayed the genes of interest, were

diploidized starting from haploids regenerated from anthers cultures, to reach the homozygosis status for all the single genes.

The target explants, roughly 30 immature embryos, were gathered six days after sampling at the center of a Petri dish containing the osmotic medium NB - with 0.4 M mannitol. After incubation for four hours the embryos were shot twice, using gold particles with a 1.5 - 3.0 micron diameter, at a pressure of 1100 psi and 27 in Hg vacuum.

Twenty four hours after the shooting the embryos were individually transferred into an NB medium and incubated for three days at 28° C in the dark, then transferred to a solid selection medium containing 50 mg/liter of hygromycin B. After two weeks of selection the embryos were transferred to an R2 liquid selection medium (Ohira et al. 1973) supplemented with 1mg/l of 2,4-D, 1 mg/l thiamine, 30 g/l saccharose and 50 mg/l hygromycin B. Embryos were maintained at 90 rpm on a rotating plate for another two weeks; the medium was changed in the middle of said period. When the hygromycin-resistant calluses became visible, they were transferred to a medium to increase the callus mass (R21) and afterwards to a regeneration medium (MS) containing 2.5 mg/l BAP and 0.5 mg/l NAA, exposed to light, with a 16-hour photoperiod. The regenerated shoots were then transferred to a radication medium for four weeks and afterwards to pots in the greenhouse.

Example 3: production of dough.

Dough was prepared using the same procedure for wheat flour (Veronese variety), rice flour (Rosa Marchetti variety) and the new flour (transgenic line PLT300R13-

7). 500 grams of flour were mixed with 350 ml of water, 10 grams of salt, 10 grams of sugar and 7 grams of dry active yeast. The dough was obtained using an autobakery and kneading the mixture for a 10-minute period. The dough was kept rising for one hour at 37°C, followed by cooking at 200° for 60 minutes.

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Table 1a

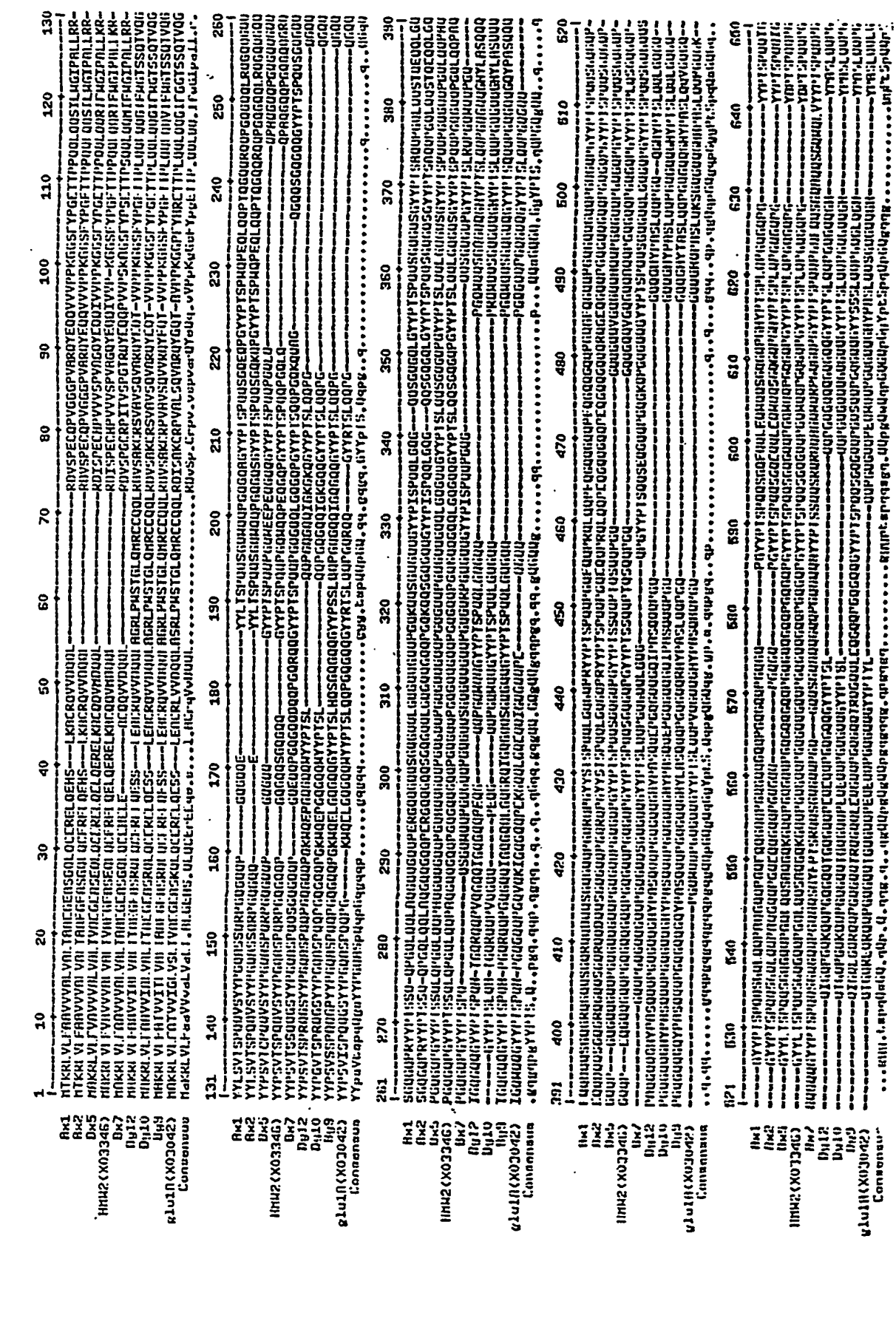

Table 1b

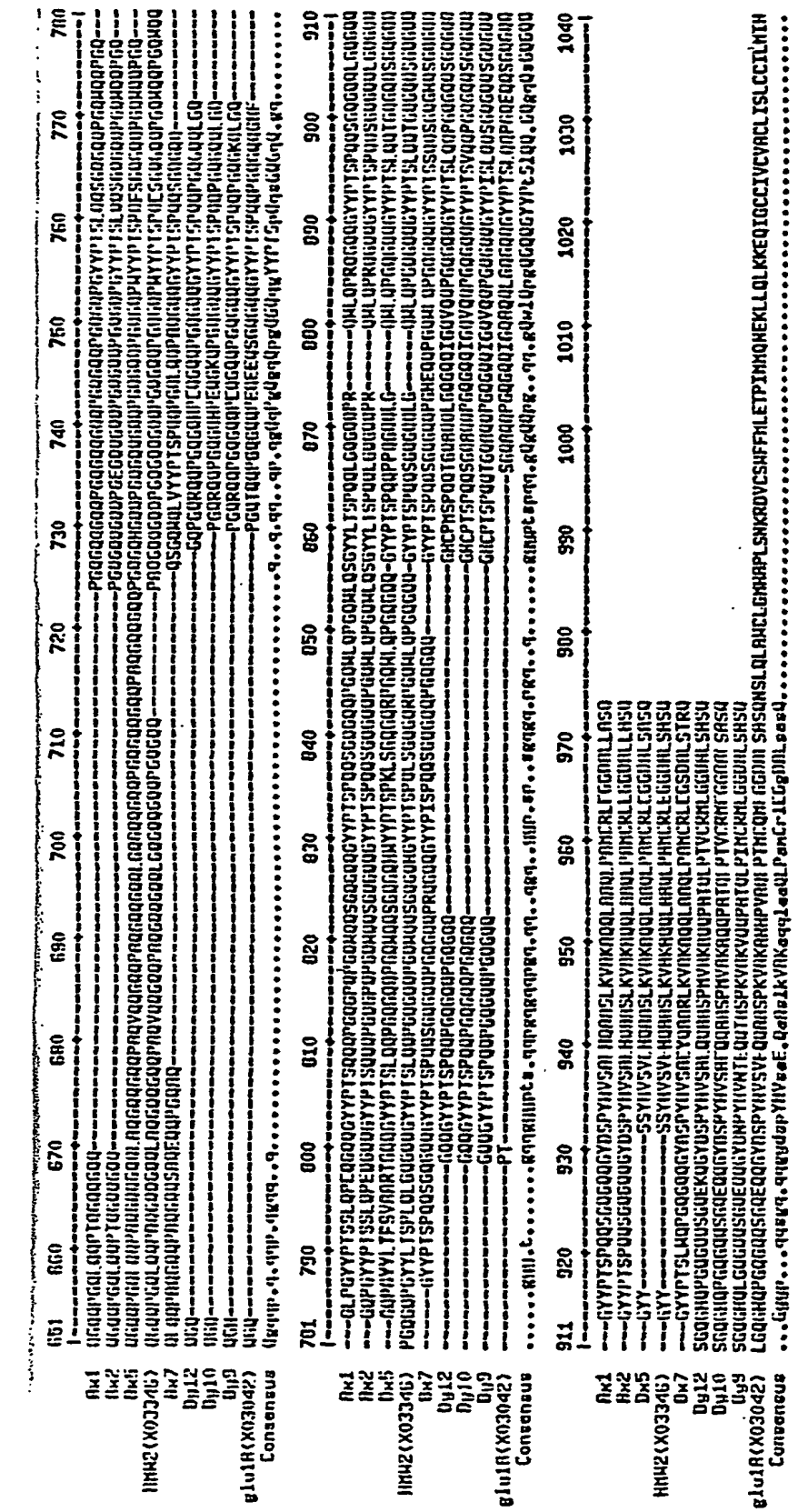

Table 2

```
1    atggcagagg atctgatcct ggagagatgt gatttgcagc tggaggtcaa
51   ggccgcgacc accgcacggc cgacctgtgc cgggagaggc tggtgttgcg
101  gcggggccag cccttctggc tgacgctgca ctttgagggc cgtggctacg
151  aggctggtgt ggacactctc accttcaacg ctgtgaccgg cccagatccc
201  agtgaggagg ccgggactat ggcccggttc tcactgtcca gtgctgtcga
251  gggggggcacc tggtcagcct cagcagtgga ccagcaggac agcactgtct
301  cgctgctgct cagcacccca gctgatgccc ccattggcct gtatcgcctc
351  agcctggagg cctccactgg ttaccagggc tccagcttcg tactgggcca
401  cttcatcctg ctctacaatc ctcggtgccc agcggatgct gtctatatgg
451  actcagacca agagcggcag gagtatgtgc tcacccaaca gggcttcatc
501  taccagggct cggccaagtt catcaatggc ataccttgga acttcgggca
551  gtttgaagat gggatcctgg atatttgcct gatgctcttg gacaccaacc
601  ccaagttcct gaagaatgct ggccaagact gctcgcgccg cagcagacct
651  gtctacgtgg gccgggtggt gagcgccatg gtcaactgca atgacgatca
701  gggcgtgctt cagggacgct gggacaacaa ctacagtgat ggtgtcagcc
751  ccatgtcctg gatcggcagc gtggacatcc tgcggcgctg gaaagactat
801  gggtgccagc gcgtcaagta cggccagtgc tgggtcttcg ctgctgtggc
851  ctgcacagtg ctgcggtgcc ttggcatccc cacccgagtc gtgaccaact
901  ttaactcagc ccacgaccag aacagcaacc tgctcatcga gtacttccga
951  aacgagtctg gggagatcga ggggaacaag agcgagatga tctggaactt
1001 ccactgctgg gtggagtcgt ggatgaccag gccggacctg gagcctgggt
1051 acgaggggtg gcaggccctg gaccccacac cccaggagaa gagtgaaggg
1101 acatactgct gtggcccagt tccggttcga gccatcaagg agggccacct
1151 gaacgtcaag tatgatgcac ctttcgtgtt tgctgaggtc aatgctgacg
1201 tggtgaactg gatccggcag aaagatgggt ccctgcgcaa gtccatcaac
1251 catttggttg tggggctgaa gatcagtact aagagtgtgg gccgcgatga
1301 gcgagaggac atcacccaca cctacaagta cccagaggga tctgaagagg
1351 agcgggaagc ttttgttagg gccaaccacc taaataaact ggccacaaag
1401 gaagaggctc aggaggaaac gggagtggcc atgcggatcc gtgtgggcca
1451 gaacatgact atgggcagtg actttgacat ctttgcctac atcaccaatg
1501 gcactgctga gagccacgaa tgccaactcc tgctctgtgc acgcatcgtc
1551 agctacaatg gagtcctggg gcccgtgtgc agcaccaacg acctgctcaa
1601 cctgaccctg gatcccttct cggagaacag catccccctg cacatcctct
1651 atgagaagta cggtgactac ctgactgagt ccaacctcat caaggtgcga
1701 ggcctcctta tcgagccagc agccaacagc tatgtattgg ccgagaggga
1751 catttacctg gagaatccag aaatcaagat ccgggtcttg ggggagccca
1801 agcagaaccg caagctgatt gctgaggtgt ctctgaagaa tccgctccct
1851 gtgccgctgc tgggttgtat cttcaccgtg gaaggagctg gcctgaccaa
1901 ggaccagaag tcggtggagg tcccagaccc cgtggaagca ggggagcaag
1951 cgaaggtacg ggtggacctg ctgccgacgg aggtgggcct ccacaagctg
2001 gtggtgaact tcgagtgcga caagctgaag gccgtgaagg gctatcggaa
2051 cgtcatcatc ggccccgcct aa
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Table 2 shows the nucleotide sequence of the gene that codes for the guinea pig transglutaminase enzyme that we cloned starting from mRNA of liver and then sequenced. The underlined bases indicate the start and stop codons.

Table 3

```
                AccI
                ApoI
                Asp700
                XmnI
                EcoRI
     1  GAATTCCTTC TACATCGGCT TAGGTGTAGC AACACGACTT TATTATTATT
        CTTAAGGAAG ATGTAGCCGA ATCCACATCG TTGTGCTGAA ATAATAATAA

                                                      BsmFI
    51  ATTATTATTA TTATTATTAT TTTACAAAAA TATAAAATAG ATCAGTCCCT
        TAATAATAAT AATAATAATA AAATGTTTTT ATATTTTATC TAGTCAGGGA

   101  CACCACAAGT AGAGCAAGTT GGTGAGTTAT TGTAAAGTTC TACAAAGCTA
        GTGGTGTTCA TCTCGTTCAA CCACTCAATA ACATTTCAAG ATGTTTCGAT

                DraI
   151  ATTTAAAAGT TATTGCATTA ACTTATTTCA TATTACAAAC AAGAGTGTCA
        TAAATTTTCA ATAACGTAAT TGAATAAAGT ATAATGTTTG TTCTCACAGT

                            NdeI
   201  ATGGAACAAT GAAAACCATA TGACATACTA TAATTTTGTT TTTATTATTG
        TACCTTGTTA CTTTTGGTAT ACTGTATGAT ATTAAAACAA AAATAATAAC

                                                      AflII
   251  AAATTATATA ATTCAAAGAG AATAAATCCA CATAGCCGTA AAGTTCTACA
        TTTAATATAT TAAGTTTCTC TTATTTAGGT GTATCGGCAT TTCAAGATGT

                AflII                                 HindIII
   301  TGTGGTGCAT TACCAAAATA TATATAGCTT ACAAAACATG ACAAGCTTAG
        ACACCACGTA ATGGTTTTAT ATATATCGAA TGTTTTGTAC TGTTCGAATC

   351  TTTGAAAAAT TGCAATCCTT ATCACATTGA CACATAAAGT GAGTGATGAG
        AAACTTTTTA ACGTTAGGAA TAGTGTAACT GTGTATTTCA CTCACTACTC

   401  TCATAATATT ATTTTCTTTG CTACCCATCA TGTATATATG ATAGCCACAA
        AGTATTATAA TAAAAGAAAC GATGGGTAGT ACATATATAC TATCGGTGTT

                                                      Alw44I
                                                      AspHI
                                                      BmyI
                                                      BsrFI-HKAI
                                                      Bsp1286I
                                                      HgiAI
                                                      SnoI
                MaeIII              EcoRV             ApaLI
   451  AGTTACTTTG ATGATGATAT CAAAGAACAT TTTTAGGTGC ACCTAACAGA
        TCAATGAAAC TACTACTATA GTTTCTTGTA AAAATCCACG TGGATTGTCT

   501  ATATCCAAAT AATATGACTC ACTTAGATCA TAATAGAGCA TCAAGTAAAA
        TATAGGTTTA TTATACTGAG TGAATCTAGT ATTATCTCGT AGTTCATTTT

   551  CTAACACTCT AAAGCAACCG ATGGGAAAGC ATCTATAAAT AGACAAGCAC
        GATTGTGAGA TTTCGTTGGC TACCCTTTCG TAGATATTTA TCTGTTCGTG

                        FokI
   601  AATGAAAATC CTCATCATCC TTCACCACAA TTCAAATATT ATAGTTGAAG
        TTACTTTTAG GAGTAGTAGG AAGTGGTGTT AAGTTTATAA TATCAACTTC

                TfiI                    MboII
   651  CATAGTAGTA GAATCCAACA ACAATGAAGA TCATTTTCGT ATTTGCTCTC
        GTATCATCAT CTTAGGTTGT TGTTACTTCT AGTAAAAGCA TAAACGAGAG

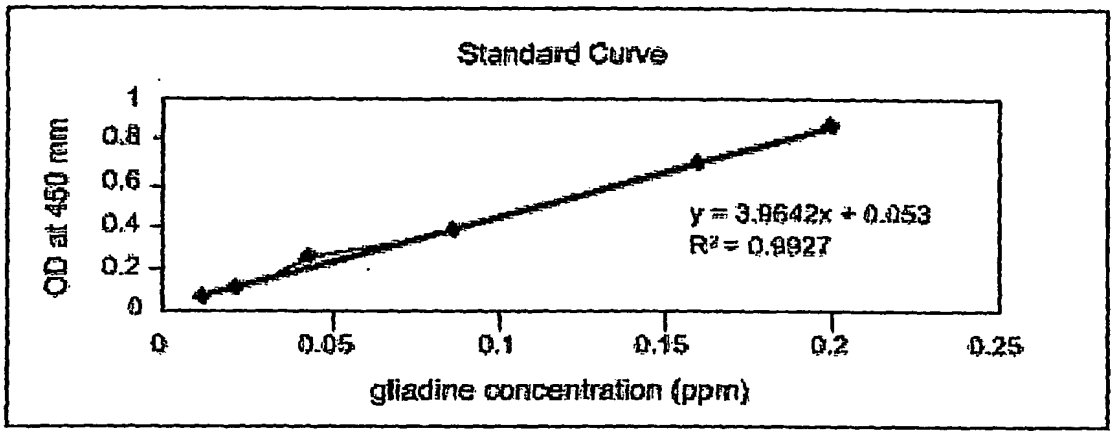
                        BsrDI           HgaI
                                                      MaeI
                SphI                                  BtsI
   701  CTTGCTATTG TTGCATGCAA TGCCTCTGCG TCTAGA
        GAACGATAAC AACGTACGTT ACGGAGACGC AGATCT
```

Table 4

Gene Name	Access Number	Sense primer Anti-sense primer	Cloning sites (5' - 3')	Amplif. Dim.
1Ax1	X61009	PLT217-GCTCAGCAGAGTTCTATCACTGGCTGGCCAAC PLT219-GGATCCGATTACGTGGCTTTAGCAGACCGTC	BamHI-PstI	2.783
1Ax2	M22208	PLT228-GGATCCGCTTAGAAGCATTGAGTGGCCGC PLT230-GCTCAGCCTATCACTGGCTGGCCAACAATGC	BamHI-CelII	2.910
1Bx7	M22209	PLT185-TCTAGAATGGCACTACTCGACATGGTTAG PLT186-CACCATGCAAGCTGCAGAGAG	XbaI-PstI	2.853
1Bx17	JC2099	PLT562-TCTAGATATGGCTAAGCGGTTAGTCCTC PLT563-GATATCTGCGAGCTGCAGAGAGTTC	XbaI-SacI	2.259
1By9	X61026	PLT272-CCCGGGCACAGATAAATGTTGTGATTCA PLT273-GTCGACTGCAAGTTGCAGAGAGTTCTAT	XbaI-SalI	2.771
1Dx5	X12928	G1B5-TGTTCCATGCAGGCTACCTCCCACTAC PLT189-GTCGACATGCCTAAGCACCATGCGAG	EcoRI-SalI	3.033
1Dy10	X12929	G2B3-AAGCTTTTCATTTTGCATTATTATTGGGTT G2B5-ACCTTATCCATGCAAGCTACCTTCCAC	EcoRI-EcoRI	2.555
1Dy12	X03041	PLT482-GAATTCGCAGATTTGCAAAAGCAATGGCTAAC PLT483-TCTAGAGCTTGTGAGAAAGGGGTAATCATCAGTG	EcoRI-PstI	3.035
HMW2	X03346	PLT488-GAATTCAGCTTTGAGTGGCCGTAGATTTGCA PLT489-GGATCCATATAGGATCTGTCGCATTCATGGCTG	EcoRi-BamHI	3.179
Glu1A	X03042	PLT571-TCTAGATGGCTAAGCGGTTGGTCCTC PLT572-GATATCGCTCCTTGTTGCATTCAACACTCTTAC	BamHI-SalI	2.895
TG	M19646	PLT237-TCTAGAATGGCAGAGGATCTGATCCTGGAG PLT238-GAGCTCTTAGGCGGGGCCGATGATGACG	XbaI-SacI	2.072

Table 5

sample	ppm	OD 450 mm	
negative control		0	0.12
STND		0.01	0.08
STND		0.02	0.12
STND		0.04	0.26
STND		0.08	0.35
STND		0.16	0.67
STND		0.2	0.86



sample	OD 450 mm	ppm		dilution	% gluten
Rice flour (Reference)		0.13	0.019	1/500	0.002
New flour		0.15	0.024	1/500	0.002
Wheat flour		0.049	0.110	1/500000	11.024

% gluten = OD (450)xFx2
F = dilution factor
2 = Total gluten conversion factor
Starting weight of the sample 5g